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(54) Title: A METHOD FOR ENHANCING AMPLIFICATION IN THE POLYMERASE CHAIN REACTION EMPLOYING PEPTIDE NUCLEIC ACID (PNA)

(57) Abstract

A process for producing a particular nucleic acid sequence from a given sequence of DNA or RNA in amounts which are large compared to the amount initially present, using PNAs in conjunction with the polymerase chain reaction (PCR) is disclosed.

A METHOD FOR ENHANCING AMPLIFICATION IN THE
POLYMERASE CHAIN REACTION EMPLOYING PEPTIDE
NUCLEIC ACID (PNA)

5   BACKGROUND OF THE INVENTION

The present invention relates to a process for
amplifying nucleic acid sequences present in a
sample. More specifically, the present invention
relates to a method of employing peptide nucleic
10   acids (PNA) to enhance the amplification of nucleic
acid sequences via the polymerase chain reaction.
Still more specifically, the present invention
relates to a process for producing a particular
nucleic acid sequence from a given sequence of DNA or
15   RNA in amounts which are large compared to the amount
initially present, using PNAs in conjunction with the
polymerase chain reaction (PCR).

The polymerase chain reaction has, since its
conception in April 1983, become a standard technique
20   for amplification of nucleic acid sequences. In this
process one or more specific nucleic acid sequences
present in a nucleic acid or mixture thereof are
amplified using primers and agents for polymerization
and then detecting the amplified sequence. The
25   extension product of one primer, when hybridized to
the other, becomes a template for the production of
the desired specific nucleic acid sequence, and vice
versa, and the process is repeated as often as is
necessary to produce the desired amount of the
30   sequence.

The DNA or RNA may be single- or double-
stranded, and may be a relatively pure species or a
component of a mixture of nucleic acids. The PCR
process utilizes a repetitive reaction to accomplish
35   the amplification of the desired nucleic acid
sequence.

Indeed, in the approximately ten years since its
discovery, the PCR technique has been widely employed
in the field of molecular biology in detecting and

- 2 -

identifying nucleic acid sequences. For example, the polymerase chain reaction (PCR) has been extensively used to amplify DNA loci in genome mapping, linkage studies, genetic diagnostics, forensics, and

5 paternity testing. The wide-spread use of this technique is testimony to the utility of the process.

Unfortunately, the polymerase chain reaction is not without problems. The PCR process depends upon multiple steps of annealing and melting (separation

10 of two complementary strands). Under ideal conditions, one can envision the separation of the two complementary strands of double-stranded DNA, annealing of the complementary primers, and elongation of the primers with a polymerase to

15 produce two molecules of double-stranded DNA. This cycle is repeated while the quantity of the desired sequence grows exponentially.

However, in most contexts in which the polymerase chain reaction is employed, situations are

20 less than ideal. Often DNA of interest to the biotechnological community contains a number of repeat or near-repeat sequences. Additionally, nucleic acid sequences frequently contain a number of internally self-complementary sequences. These

25 repeating and self-complementary sequences increase the likelihood that the nucleic acid sequence will experience interstrand and intrastrand interactions, possibly resulting in a nucleic acid strand folding in upon itself.

30 Further, as the polymerase chain reaction progresses, the concentration of complementary DNA sequences increases exponentially, while the concentration of primer commonly remains constant or decreases. This can result in the product of the

35 interim cycles of the polymerase chain reaction

- 3 -

successfully competing with th primers required for
the next cycle of elongation. Such competition can
significantly reduce the efficiency and yield of the
polymerase chain reaction.

5   Moreover, diploid DNA poses additional
challenges. Robust amplification of allelic
sequences is often difficult, particularly when there
is a significant size difference between the two
alleles. Smaller alleles simply amplify more

10   efficiently. This phenomenon, known as differential
amplification, results in the generation of more
copies of the smaller allele and thus a more intense
band on the subsequent electrophoresis gel. At best,
the larger allele is underrepresented relative to the

15   smaller allele, at worst, differential amplification
can result in allelic dropout, in which the larger
allele amplifies so poorly relative to the smaller
allele that the larger allele can neither be
visualized nor detected. Differential amplification

20   and allelic dropout can complicate genetic analyses.
        The terms "peptide nucleic acid" and "PNA" refer
to a DNA analog with a backbone consisting of N-(2-
aminoethyl)glycine units. To this backbone,
analogous to DNA, are attached the nucleobases - for

25   DNA, adenine, guanine, cytidine, and thymine. The
individual monomeric units of PNA can be synthesized
to furnish a PNA chain having a specific sequence of
bases. The synthesis of such PNA chains is detailed
in various publications, including Science 254, 1497

30   (1991); J. Am. Chem. Soc. 114, 9677 (1992); J. Am.
Chem. Soc. 144, 1895 (1992); J. Chem. Soc. Chem.
Comm. 800 (1993); Proc. Nat. Acad. Sci. USA 90, 1667
(1993); Intercept Ltd. 325 (1992); J. Am. Chem. Soc.
114, 9677 (1992); Nucleic Acids Res. 21, 197 (1993);

35   J. Chem. Soc. Chem. Commun. 518 (1993); Anti-Cancer

- 4 -

Drug Design 8, 53 (1993); Nucleic Acids Res. 21, 2103
(1993); Org. Proc. Prep. 25, 457 (1993); CRC Press
363 (1992); J. Chem. Soc. Chem. Commun. 800 (1993);
J. Am. Chem. Soc. 115, 6477 (1993); Nature 365, 566
5   (1993); ABRF News Vol. 4, No. 3 (1993); Science 258,
1481 (1992); WO 8-92/20702; and WO 92/20703, the
contents of which are incorporated herein by
reference. Additionally, specific sequences of PNA
are commercially available from BioSearch Div.,
10   PerSeptive Biosystems, Inc., Bedford, MA.
        PNA has been demonstrated to be a potent DNA
mimic in terms of sequence-specific annealing.
Experimental results (discussed in the above
publications) have demonstrated that at physiological
15   ionic strength a PNA/DNA duplex is generally 1° C per
base pair more stable thermally than the
corresponding DNA/DNA duplex. Other experimental
data has indicated that PNA is more stable in the
cell than DNA; that PNA binds to DNA or RNA 50-100
20   times more tightly than either DNA or RNA; that PNA
can invade and displace double-stranded DNA (dsDNA);
and that the backbone of PNA adopts a helical
conformation. However, a single base mismatch in a
PNA/DNA duplex is much more destablizing than a
25   mismatch in the corresponding DNA/DNA duplex.
Furthermore, PNA does not function as a primer for
DNA polymerase.
        Recently, PNAs have been used to detect single
base mutations through PCR clamping. In Nucleic
30   Acids Research 21, 5332 (1993), PNAs were reported to
form a PNA/DNA complex which effectively blocked the
formation of a PCR product. However, this PNA-
directed PCR clamping results in a blocking of PCR
amplification, rather than an enhancement of the
35   polymerase chain reaction.

- 5 -

Accordingly, there remains a need for an
efficient and effective method of amplifying existing
nucleic acid sequences present in a sample using the
polymerase chain reaction.

5   SUMMARY OF THE INVENTION

Accordingly, an object of the present invention
is to provide a method for enhancing amplification of
nucleic acid sequences using the polymerase chain
reaction.

10   Another object of the present invention is to
provide a kit for detecting a nucleic acid sequence
of interest.

Still another object of the present invention is
to provide a method of cloning into a vector a

15   nucleic acid sequence of interest.

Still another object of the present invention is
to provide an amplified nucleic acid sequence.

Still another object of the present invention is
to provide a method for replicating a target nucleic

20   acid strand with a polymerase to produce a
complementary nucleic acid strand.

Still another object of the present invention is
to provide a method for replicating a target nucleic
acid strand with a thermostable DNA polymerase to

25   produce a complementary nucleic acid strand.

Still another object of the present invention is
to provide a method for replicating a target nucleic
acid strand with a polymerase.

These and other objects of the present invention

30   are achieved by means of a process for detecting the
presence or absence of at least one nucleic acid
sequence of interest in a sample containing a nucleic
acid or a mixture of nucleic acids, comprising
treating the sample with at least one PNA oligomer

35   which binds within the region of nucleic acid to be

- 6 -

amplified. More specifically, the present invention
resides in a process for amplifying one or more
specific nucleic acid sequences present in a nucleic
acid or mixture thereof using oligonucleotide primers

5   and an agent for polymerization, employing at least
one PNA oligomer to reduce the incidence of
interstrand and intrastrand interactions, and then
isolating and/or detecting the amplified sequence.
This method is expected to be more efficient and

10   effective than the conventional PCR technique for
producing large amounts of nucleic acid from a target
sequence, especially with DNA or RNA that contains
one or more repeat or near-repeat sequences.
More specifically, the present invention

15   provides a process for detecting the presence or
absence of at least one nucleic acid sequence of
interest in a sample containing a nucleic acid or
mixture of nucleic acids, wherein the sample is
suspected of containing said sequence or sequences,

20   which process comprises the steps of treating the
sample with at least one PNA oligomer which binds
within the nucleic acid sequence to be amplified; and
polymerizing a nucleic acid sequence which is
complementary to the nucleic acid sequence of

25   interest.
In another preferred embodiment of the present
invention, a method of amplifying a nucleic acid
sequence of interest in a sample containing at least
one nucleic acid, comprises: (a) treating the sample

30   with (i) an agent for polymerization, (ii) at least
one oligonucleotide primer which binds to a strand of
each different nucleic acid sequence of interest,
(iii) at least one PNA oligomer which is
substantially complementary to a portion of and which

35   binds within the nucleic acid sequenc of int rest at

- 7 -

a position different from the position at which the
oligonucleotide primer binds, and (iv) four different
nucleoside triphosphates, forming a first mixture;
(b) heating the first mixture to within a first
5   temperature range so as to denature the nucleic acid
and to separate any of the at least one
oligonucleotide primer and any of the at least one
PNA oligomer which is bound to the nucleic acid,
forming a second mixture; (c) cooling the second
10   mixture to within a second temperature range at which
the at least one PNA oligomer binds to the nucleic
acid sequence of interest, forming a third mixture;
(d) bringing the third mixture to within a third
temperature range at which the at least one
15   oligonucleotide primer binds to the nucleic acid
sequence of interest and at which an extension
product is synthesized from the oligonucleotide
primer on each strand, provided the nucleic acid
sequence of interest is present, said synthesis
20   employing the nucleic acid as a template, and whereby
the at least one PNA oligomer is displaced from the
nucleic acid sequence of interest during said
synthesis.

In still another preferred embodiment, the
25   present invention relates to a kit for detecting at
least one nucleic acid sequence of interest in a
sample, comprising: (a) a container containing at
least one oligonucleotide primer for each strand of
each nucleic acid sequence of interest, each of said
30   at least one oligonucleotide primer being
substantially complementary to a strand of each
nucleic acid sequence of interest; (b) a container
containing at least one PNA oligomer which is
substantially complementary to a portion of and which
35   binds within each nucleic acid sequence of interest

- 8 -

at a position different from the position at which
the at least one oligonucleotide primer binds; and
(c) a container containing each of four different
nucleoside triphosphates.

5   In still another preferred embodiment, the
present invention pertains to a process for cloning
into a vector a nucleic acid sequence of interest in
a sample containing at least one nucleic acid,
comprising: (a) treating the sample with (i) an agent
10   for polymerization, (ii) at least one oligonucleotide
primer containing a restriction site at its 5' end
and which is substantially complementary to and binds
to a strand of each different nucleic acid sequence
of interest, (iii) at least one PNA oligomer which is
15   substantially complementary to a portion of and which
binds within the nucleic acid sequence of interest at
a position different from the position at which the
oligonucleotide primer binds, and (iv) four different
nucleoside triphosphates, forming a first mixture;
20   (b) heating the first mixture to within a first
temperature range so as to denature the nucleic acid
and to separate any of the at least one
oligonucleotide primer and any of the at least one
PNA oligomer which is bound to the nucleic acid,
25   forming a second mixture; (c) cooling the second
mixture to within a second temperature range at which
the at least one PNA oligomer binds to the nucleic
acid, forming a third mixture; (d) bringing the third
mixture to within a third temperature range at which
30   the at least one oligonucleotide primer binds to the
nucleic acid sequence of interest and at which an
extension product is synthesized from the
oligonucleotide primer on each strand, provided the
nucleic acid sequence of interest is present, said
35   synthesis employing the nucleic acid as a template,

- 9 -

and whereby the at least one PNA oligomer is
displaced from the nucleic acid sequence of interest
during said synthesis (e) adding to the product of
step (d) a restriction enzyme which cleaves at said
restriction site to thereby obtain cleaved products;
and (f) ligating the cleaved products into at least
one cloning vector.

The steps detailed above may be repeated until
the desired level of sequence amplification is
obtained.

In still another preferred embodiment, the
present invention pertains to a method for
replicating a target nucleic acid strand with a
polymerase to produce a complementary nucleic acid
strand, comprising:(A) providing a peptide nucleic
acid (PNA) which, (1) is substantially complementary
in sequence to nucleobases in at least a portion of
the target strand, (2) anneals with said portion of
the target strand, and (3) when annealed with said
portion of the target strand, does not serve as a
replication initiation site for the polymerase; and
(B) annealing the PNA to the target strand prior to
initiating replication thereby to enhance replication
by the polymerase during production of the
complementary nucleic acid strand.

In still another preferred embodiment, the
present invention pertains to a method for
replicating a target nucleic acid strand with a
thermostable DNA polymerase to produce a
complementary nucleic acid strand, comprising: (A)
providing a peptide nucleic acid (PNA) which, (1) is
substantially complementary in sequence to
nucleobases in at least a portion of the target
strand, (2) anneals with said portion of the target
strand, and (3) when annealed with said portion of

- 10 -

the target strand, does not serve as a replication
initiation site for the polymerase; and (B) annealing
the PNA to the target strand prior to initiating
replication by the polymerase thereby to enhance
5   replication by the polymerase during production of
the complementary nucleic acid strand.

In still another preferred embodiment, the
present invention pertains to a method for
replicating a target nucleic acid strand with a
10 polymerase comprising: (A) providing a target nucleic
acid strand having a multiplicity of nucleobase
sequence repeats; (B) annealing a PNA to the target
strand which: (1) is substantially complementary in
sequence to at least a portion of one of the repeats;
15 (2) anneals with the at least a portion of one of the
repeats thereby to form a target strand-PNA complex;
and (3) when annealed with the at least a portion of
one of the repeats, does not serve as a replication
initiation site for the polymerase; and (C)
20 incubating the complex in the presence of a
polymerase, such that the polymerase enhances
replication of the target strand as compared with
when the target strand is not complexed with the PNA.

BRIEF DESCRIPTION OF THE DRAWINGS

25   A more complete appreciation of the invention
and many of the attendant advantages thereof will be
readily obtained as the same become better understood
by reference to the following detailed description
when considered in connection with the accompanying
30 drawings, wherein:

Figure 1 provides a schematic illustration of
the conventional polymerase chain reaction.

Figure 2 illustrates the chemical structure of
the monomeric subunits of PNA and deoxyribonucleic

- 11 -

acid (DNA), where B is the nucleobase and R^1 is either
a hydrogen atom or lysine amide.

Figure 3 shows a naturally occurring
deoxyribooligonucleotide oligomer (A) and PNA

5   oligomer (B).

Figure 4 provides a schematic illustration of
the PNA-enhanced polymerase chain reaction.

Figure 5 is an autoradiograph illustrating the
results of a polymerase chain reaction obtained in

10   the absence of a PNA oligomer and with varying
concentrations of a PNA oligomer.

Figure 6 is an autoradiograph illustrating the
results of the PNA-enhanced polymerase chain reaction
and the conventional PCR run with various samples of

15   DNA.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

In an attempt to enhance the amplification of
nucleic acid sequences using the polymerase chain
reaction, the program of research directed to

20   diminishing the interstrand and intrastrand
interactions which decrease the efficiency of the PCR
reaction was undertaken. That investigation resulted
in the discovery that PNA oligomers, which are
substantially complementary to a portion of the

25   nucleic acid sequence of interest but different from
that portion of the nucleic acid sequence to which
the oligonucleotide primers bind, apparently
sufficiently block the nucleic acid functioning as a
template, such that the template is unavailable for

30   interactions, either interstrand or intrastrand,
while allowing amplification of the sequence to
proceed.

The proposed mechanism will become apparent in
the following exemplary comparison of the

- 12 -

conventional polymerase chain reaction and the
PNA-enhanced PCR reaction.

In the conventional PCR reaction, as illustrated
in Figure 1, a double-stranded DNA molecule, for
5   example, containing the desired sequence comprised of
complementary strands is utilized as the nucleic
acid. The first and each subsequent reaction cycle
extension of each oligonucleotide primer on the
original template will produce one new ssDNA molecule
10   product of indefinite length which terminates with
only one of the primers. These products, referred to
as "long products," will accumulate in a linear
fashion; that is, the amount present after any number
of cycles will be proportional to the number of
15   cycles.

The long products thus produced will act as
templates for one or the other of the oligonucleotide
primers and the at least one PNA oligomer during
subsequent cycles and will produce molecules of the
20   desired sequence. These molecules will also function
as templates for one or the other of the
oligonucleotide primers and the at least one PNA
oligomer, producing additional amounts of the desired
sequence. Thus a chain reaction can be sustained
25   which will result in the accumulation of the desired
sequence at an exponential rate relative to the
number of cycles.

In the PNA-enhanced PCR reaction, as illustrated
in Figure 4, a DNA sequence of interest is placed in
30   solution with two oligonucleotide primers, one
oligonucleotide primer for each strand of each
nucleic acid sequence of interest, one or more PNA
oligomers which bind within the region of nucleic
acid to be amplified but in a position different from
35   that which the oligonucleotide primers bind, a

- 13 -

polymerase, and a mixture of four nucleoside
triphosphates, deoxyadenosine triphosphate
deoxythymidine triphosphate, deoxyguanosine
triphosphate, and deoxycytidine triphosphate. The
5   mixture is heated to denature the double-stranded
nucleic acid. Such denaturing can be achieved
thermally, usually at a temperature of 80° to 100° C,
preferably 95° ± 5° C. It is not necessary to
maintain the mixture at the denaturing temperature
10   for any significant length of time. Indeed, in one
embodiment of the present invention, the mixture
merely reaches the denaturing temperature range and
then it is cooled to the PNA oligomer annealing
temperature range. Accordingly, a suitable length of
15   time for maintaining this temperature is between an
instantaneous period of time and 10 minutes.
        The mixture is cooled to allow the one or more
PNA oligomers to anneal. The PNA annealing step can
occur at any temperature between the denaturing
20   temperature of the double-stranded DNA and the primer
annealing temperature. A suitable temperature for
the PNA annealing step is a range of from about 70°
to 80°C, preferably 72° to 78°C. It is not necessary
to maintain the mixture at the PNA annealing
25   temperature for any significant length of time.
Indeed, in one embodiment of the present invention,
the mixture merely passes through the PNA annealing
temperature range as it is cooled to the primer
annealing temperature range. Accordingly, a suitable
30   range of time for maintaining the PNA annealing
temperature is from an instantaneous period of time
up to ten minutes.
        The mixture is then brought to a temperature at
which the oligonucleotide primers anneal to the DNA
35   template and an extension product from the primers

- 14 -

using the nucleic acid sequence as a template is
synthesized. A suitable temperature for the for the
primer annealing and primer extension product
synthesis step is 40° to 80° C. The mixture is

5   maintained at the primer annealing and primer
extension product synthesis step for a period of time
ranging from an instantaneous period of time to 30
minutes.

      These steps constitute one cycle of the

10   PNA-enhanced PCR reaction. To initiate the next
cycle, the resultant product of the reaction, two
double-stranded DNA molecules, are denatured as in
the first step of this cycle.

      In a preferred embodiment of the present

15   invention, the oligonucleotide annealing step and the
extension product synthesis step are carried out at
two separate temperature ranges. The mixture
resulting from annealing of the at least one PNA
oligomer is brought to a temperature at which the

20   oligonucleotide primers anneal to the DNA template.
A suitable temperature for the primer annealing step
is 40° to 72°C. The mixture is maintained at the
primer annealing temperature for a period of time
ranging from an instantaneous period of time to 5

25   minutes.

      After the primers have annealed to the DNA
templates, the mixture is heated to the primer
extension product synthesis temperature. Preferably,
this primer extension product synthesis temperature

30   ranges from 60° - 80° C. The mixture is maintained
at the primer extension product synthesis temperature
for a period of time ranging from an instantaneous
period of time to 10 minutes.

      It is postulated that, upon denaturation of the

35   doubl -stranded DNA sequence of interest and cooling

- 15 -

to the PNA annealing temperature, the one or more PNA
oligomers bind to the complementary regions of the
DNA at a region of the nucleic acid sequence which is
different from those regions to which the
5   oligonucleotide primers bind. This binding of the
one or more PNA oligomers diminishes or eliminates
the annealing of the DNA, either internally with a
self-complementary region or externally with a
complementary strand of DNA. Upon further cooling to
10   the oligonucleotide primer annealing temperature, the
primers bind to the DNA templates. Because the PNA
oligomers bind to different sites than those to which
the oligonucleotide primers bind, the PNA annealing
does not interfere with primer annealing.
15   Upon subsequent warming to the polymerization
temperature, the primers are extended by a
polymerase. This extension of the primer results in
displacement of the one or more PNA oligomers,
allowing the synthesis of an extension product
20   through the region to which the one or more PNA
oligomers were bound. This displacement of the one
or more PNA oligomers during extension of the primer
is presumed to result from displacement of the PNA
oligomer by the primer extension product, possibly
25   augmented by partial thermal denaturation of the
DNA/PNA complex.
            The PNAs are synthesized by adaptation of
standard peptide synthesis procedures, either in
solution or on a solid phase. The synthesis of PNAs
30   is described in Science 254, 1497 (1991); J. Am.
Chem. Soc. 114, 9677 (1992); J. Am. Chem. Soc. 144,
1895 (1992); J. Chem. Soc. Chem. Comm. 800 (1993);
Proc. Nat. Acad. Sci. USA 90, 1667 (1993); Intercept
Ltd. 325 (1992); J. Am. Chem. Soc. 114, 9677 (1992);
35   Nucleic Acids Res. 21, 197 (1993); J. Chem. Soc.

- 16 -

Chem. Commun. 518 (1993); Anti-Cancer Drug Design 8, 53 (1993); Nucleic Acids Res. 21, 2103 (1993); Org. Proc. Prep. 25, 457 (1993); CRC Press 363 (1992); J. Chem. Soc. Chem. Commun. 800 (1993); J. Am. Chem. Soc. 115, 6477 (1993); Nature 365, 566 (1993); ABRF News Vol. 4, No. 3 (1993); Science 258, 1481 (1992); WO 8-92/20702; and WO 92/20703, the contents of which are incorporated herein by reference.

The present invention comprises a method using PNA to enhance the efficiency of the PCR amplification of nucleic acid sequences. If present during PCR amplification, PNA allows fragments of different size to be more effectively and more evenly amplified. Differential amplification is less apparent and as a result the risk of misclassification is greatly reduced. In addition, fragments of same or similar size are also amplified more effectively.

The one or more PNA oligomers are selected such that blocking of the nucleic acid template occurs without halting the PCR reaction. The PNA molecule must anneal to the DNA template before the complementary DNA strand. However, it is preferred that the PNA be sufficiently destablized at the primer extension temperature to allow the polymerase to dislodge it and extend through to the end of the template.

The term "PNA oligomer" as used herein is defined as a molecule comprised of two or more PNA monomers. Its exact size will depend on many factors, which in turn depend on the ultimate function or use of the PNA oligomer. In one preferred embodiment of the present invention, the PNA oligomer comprises 5 - 20 PNA monomers, and more preferably 8 - 16 PNA monomer units.

- 17 -

Additionally, the sequence of the PNA oligomer is designed to be substantially complementary to a nucleic acid sequence. This means that the one or more PNA oligomers must be sufficiently complementary

5 as to anneal with their respective complementary strands. Therefore, the sequence of PNA oligomer need not reflect the exact sequence of the template. Non-complementary bases or longer sequences can be interspersed in the sequence, provided that the

10 sequence has sufficient complementarity with the sequence of the strand to be amplified to hybridize therewith. Within the context of a preferred embodiment, substantial complementarity of a PNA oligomer designates a situation in which there is not

15 more than two mismatches between the nucleobase sequence of the PNA oligomer and the complementary DNA sequence.

The term "oligonucleotide" as used herein in referring to primers is defined as a molecule

20 comprised of two or more deoxyribonucleotides or ribonucleotides, preferably more than three. Its exact size will depend on many factors, which in turn depend on the ultimate function or use of the oligonucleotide.

25 The term "primer" as used herein refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed in conditions

30 such that synthesis of a primer extension product which is complementary to a nucleic acid strand is induced, i.e., in the presence of nucleotides and an agent for polymerization, such as a DNA polymerase, and at a suitable temperature and pH. The primer is

35 preferably single-stranded for maximum efficiency in

- 18 -

amplification, but may alternatively be double-
stranded. If double-stranded, the primer is first
treated to separate its strands before being used to
prepare extension products. Preferably, the primer
5   is an oligodeoxyribonucleotide. The primer must be
sufficiently long to prime the synthesis of extension
products in the presence of the agent for
polymerization. The exact lengths of the primers
will depend on many factors, including temperature
10   and the source of the primer. For example, depending
on the complexity of the target sequence, the
oligonucleotide primer typically contains 15-25 or
more nucleotides, although it may contain fewer
nucleotides. Short primer molecules generally
15   require cooler temperatures to form sufficiently
stable complexes with the template.

The primers are selected to be "substantially"
complementary to one or both strands of each specific
sequence to be amplified. This means that the
20   primers must be sufficiently complementary to anneal
with their respective strands. Therefore, the
sequence need not reflect the exact sequence of the
template. For example, a non-complementary
nucleotide fragment may be attached to the 5' end
25   with the remainder of the sequence being
complementary to the strand. Alternatively, non-
complementary bases or longer sequences can be
interspersed in the sequence, provided that the
sequence has sufficient complementarity with the
30   sequence of the strand to be amplified to hybridize
therewith and thereby form a template for synthesis
of the extension product of the other primer.

As used herein, the terms "restriction
endonucleases" and "restriction enzymes" refer to

- 19 -

bacterial enzymes each of which cut double-stranded
DNA at or near a specific nucleotide sequence.

As used herein, the term "DNA polymorphism"
refers to the condition in which two or more
5   different nucleotide sequences coexist in the same
interbreeding population in a DNA sequence.

The present invention is directed to a process
for amplifying one or more desired specific nucleic
acid sequences suspected of being in a nucleic acid.
10   Because large amounts of a specific sequence may be
produced by this process, the present invention may
be used for improving the efficiency of cloning DNA
or messenger RNA and for amplifying a target sequence
to facilitate detection thereof.
15   In general, the present process involves a chain
reaction for producing, in exponential quantities
relative to the number of reaction steps involved, at
least one specific nucleic acid sequence given that
the ends of the required sequence are known in
20   sufficient detail that oligonucleotide primers can be
synthesized which will anneal to the ends, and that
at least a portion of the sequence different from the
ends to which the primers bind is known in sufficient
detail that one or more PNA oligomers can be
25   synthesized which will anneal to the sequence. The
product of the chain reaction will be a discrete
nucleic acid duplex with termini corresponding to the
ends of the specific primers employed.

Any source of nucleic acid, in purified or
30   nonpurified form, can be utilized as the starting
nucleic acid or acids, provided it is suspected of
containing the specific nucleic acid sequence
desired. Thus, the process may employ, for example,
DNA or RNA, including messenger RNA, which DNA or RNA
35   may be single-stranded or double-stranded. In

- 20 -

addition, a DNA-RNA hybrid which contains one strand of each may be utilized. A mixture of any of these nucleic acids may also be employed, or the nucleic acids produced from a previous amplification reaction
5   herein using the same or different primers may be so utilized. The specific nucleic acid sequence to be amplified may be only a fraction of a larger molecule or can be present initially as a discrete molecule, so that the specific sequence constitutes the entire
10 nucleic acid. It is not necessary that the sequence to be amplified be present initially in a pure form; it may be a minor fraction of a complex mixture, such as a portion of the β-globin gene contained in whole human DNA or a portion of nucleic acid sequence due
15 to a particular microorganism which organism might constitute only a very minor fraction of a particular biological sample. The starting nucleic acid may contain more than one desired specific nucleic acid sequence which may be the same or different.
20 Therefore, the present process is useful not only for producing large amounts of one specific nucleic acid sequence, but also for amplifying simultaneously more than one different specific nucleic acid sequence located on the same or different nucleic acid
25 molecules.

        The nucleic acid or acids may be obtained from any source, for example, from plasmids such as pBR322, from cloned DNA or RNA, or from natural DNA or RNA from any source, including bacteria, yeast,
30 viruses, and higher organisms, such as plants or animals. DNA or RNA may be extracted from blood or tissue material, such as chorionic villi or amniotic cells, by a variety of techniques such as that described by Maniatis et al., Molecular Cloning: A

- 21 -

Laboratory Manual, (New York: Cold Spring Harbor
Laboratory, 1982), pp. 280-281.

Any specific nucleic acid sequence can be
produced by the present process. It is only
5   necessary that a sufficient number of bases at both
ends of the sequence and at some point intermediate
to the two ends of the sequence be known in
sufficient detail so that two oligonucleotide primers
and one or more PNA oligomers can be prepared which
10 will anneal to the strands of the desired sequence
and at relative positions along the sequence such
that an extension product synthesized from one
primer, when it is separated from its template
(complement), can serve as a template for extension
15 of the other primer into a nucleic acid of defined
length. The greater the knowledge about the bases at
both ends of the sequence and at the intermediate
point, the greater can be the specificity of the
primers and the PNA oligomers for the target nucleic
20 acid sequence, and thus the greater the efficiency of
the process.

It will be understood that the word
oligonucleotide primer and PNA oligomer as used
hereinafter may refer to more than one
25 oligonucleotide primer or PNA oligomer, particularly
in the case where there is some ambiguity in the
information regarding the terminal sequences or
intermediate sequence of the fragment to be
amplified. For instance, in the case where a nucleic
30 acid sequence is inferred from protein sequence
information a collection of primers and oligomers
containing sequences representing all possible codon
variations based on degeneracy of the genetic code
will be used for each strand.

- 22 -

The oligonucleotide primers may be prepared
using any suitable method, such as, for example, the
phosphotriester and phosphodiester methods described
above, or automated embodiments thereof. In one such
5 automated embodiment, diethylphosphoramidites are
used as starting materials and may be synthesized as
described by Beaucage et al., Tetrahedron Letters **22**,
1859-1862 (1981). One method for synthesizing
oligonucleotides on a modified solid support is
10 described in U.S. Pat. No. 4,458,066. It is also
possible to use a primer which has been isolated from
a biological source, such as a restriction
endonuclease digest.

The specific nucleic acid sequence is produced
15 by using the nucleic acid containing that sequence as
a template. If the nucleic acid contains two
strands, it is necessary to separate the strands of
the nucleic acid before it can be used as the
template, either as a separate step or simultaneously
20 with the synthesis of the primer extension products.
This strand separation can be accomplished by any
suitable denaturing method including physical,
chemical or enzymatic means. One physical method of
separating the strands of the nucleic acid involves
25 heating the nucleic acid until it is completely
(> 99%) denatured. Typical heat denaturation may
involve temperature ranging from about 80° to 100° C
for times ranging from an instantaneous period of
time to 10 minutes. Strand separation may also be
30 induced by an enzyme from the class of enzymes known
as helicases or the enzyme RecA, which has helicase
activity and is known, in the presence of riboATP, to
denature DNA. The reaction conditions suitable for
separating the strands of nucleic acids with
35 helicases are described by Cold Spring Harbor

- 23 -

Symposia on Quantitative Biology, Vol. XLIII "DNA:
Replication and Recombination" (New York: Cold Spring
Harbor Laboratory, 1978), B. Kuhn et al., "DNA
Helicases", pp. 63-67, and techniques for using RecA
5   are reviewed in C. Radding, Ann. Rev. Genetics,
16:405-37 (1982).

Preferably, a molar excess, usually about $10^{14}:1$
primer:template of the two oligonucleotide primers is
added to the buffer containing the separated template
10   strands. It is understood, however, that the amount
of complementary strand may not be known if the
process herein is used for diagnostic applications,
so that the amount of primer relative to the amount
of complementary strand cannot be determined with
15   certainty. As a practical matter, however, the
amount of primer added will generally be in molar
excess over the amount of complementary strand
(template) when the sequence to be amplified is
contained in a mixture of complicated long-chain
20   nucleic acid strands. A large molar excess is
preferred to improve the efficiency of the process.

If the original nucleic acid containing the
sequence to be amplified is single-stranded, its
complement is synthesized by adding one or two
25   oligonucleotide primers thereto. If an appropriate
single primer is added, a primer extension product is
synthesized in the presence of the primer, an agent
for polymerization and the four nucleotides described
below. The product will be partially complementary
30   to the single-stranded nucleic acid and will anneal
with the nucleic acid strand to form a duplex of
unequal length strands which may then be separated
into single strands as described above to produce two
single separated complementary strands.
35   Alternatively, two appropriate primers may be added

- 24 -

to the single-stranded nucleic acid and the reaction carried out.

If the original nucleic acid constitutes the sequence to be amplified, the primer extension

5 products produced will be completely complementary to the strands of the original nucleic acid and will anneal therewith to form a duplex of equal length strands to be separated into single-stranded molecules.

10 Preferably, a molar excess, usually about $10^{14}:1$ primer:template of the PNA oligomer is added to the buffer containing the template. It is understood, however, that the amount of complementary strand may not be known if the process herein is used for

15 diagnostic applications, so that the amount of PNA oligomer relative to the amount of complementary strand cannot be determined with certainty. As a practical matter, however, the amount of PNA oligomer added will generally be in molar excess over the

20 amount of complementary strand (template) when the sequence to be amplified is contained in a mixture of complicated long-chain nucleic acid strands. A large molar excess is preferred to improve the efficiency of the process.

25 When the complementary strands of the nucleic acid or acids are separated, whether the nucleic acid was originally double or single-stranded, the strands are ready to be used as a template for the synthesis of additional nucleic acid strands. This synthesis

30 can be performed using any suitable method. Generally it occurs in a buffered aqueous solution, preferably at a pH of 7-9, most preferably about 8. The deoxyribonucleoside triphosphates dATP, dCTP, dGTP and TTP are also added to the synthesis

35 mixture in adequate amounts and the resulting

- 25 -

solution is heated to about 90° - 100° C. for from an instaneous period of time to 10 minutes, preferably from 1 to 4 minutes. After this heating period the solution is allowed to cool to from 40° - 80° C, 5 which is preferable for the primer hybridization. To the cooled mixture is added an agent for polymerization, and the reaction is allowed to occur under conditions known in the art. This synthesis reaction may occur at from room temperature up to a 10 temperature above which the agent for polymerization no longer functions efficiently. Thus, for example, if heat-stable DNA polymerase is used as the agent for polymerization, the temperature is generally no greater than about 72° C.

15   The agent for polymerization may be any compound or system which will function to accomplish the synthesis of primer extension products, including enzymes. Suitable enzymes for this purpose include, for example, Taq polymerase, AMPLITAQ DNA polymerase 20 (available from Perkin Elmer-Cetus), E. coli DNA polymerase I, Klenow fragment of E. coli DNA polymerase I, T4 DNA polymerase, other available DNA polymerases, reverse transcriptase, and other enzymes, including heat-stable enzymes, which will 25 facilitate combination of the nucleotides in the proper manner to form the primer extension products which are complementary to each nucleic acid strand. Generally, the synthesis will be initiated at the 3' end of each primer and proceed in the 5' to 3' 30 direction along the template strand, until synthesis terminates, producing molecules of different lengths. There may be agents, however, which initiate synthesis at the 5' end and proceed in the other direction, using the same process as described above.

- 26 -

The newly synthesized strand and its complementary nucleic acid strand form a double-stranded molecule which is used in the succeeding steps of the process. In the next step, the strands

5 of the double-stranded molecule are separated using any of the procedures described above to provide single-stranded molecules.

New nucleic acid is synthesized on the single-stranded molecules. Additional agent for

10 polymerization, nucleotides, PNA oligomers, and oligonucleotide primers may be added, if necessary, for the reaction to proceed under the conditions prescribed above. Again, the synthesis will be initiated at one end of the oligonucleotide primer

15 and will proceed along the single strands of the template to produce additional nucleic acid. After this step, half of the extension product will consist of the specific nucleic acid sequence bounded by the two primers.

20 The steps of strand separation, PNA oligomer annealing, oligonucleotide primer annealing, and extension product synthesis can be repeated as often as needed to produce the desired quantity of the desired nucleic acid sequence. The amount of the

25 nucleic acid sequence produced will accumulate in an exponential fashion.

When it is desired to produce more than one nucleic acid sequence from the first nucleic acid or mixture of nucleic acids, the appropriate number of

30 different oligonucleotide primers and PNA oligomers are utilized. For example, if two different specific nucleic acid sequences are to be produced, four primers and two or more PNA oligomers are utilized. Two of the primers are specific for one of the

35 nucleic acid sequences and the other two primers are

specific for the second nucleic acid sequence. At
least one of the PNA oligomers is specific for each
nucleic acid sequence. In this manner, each of the
two different specific sequences can be produced
5   exponentially by the present process.

The present invention can be performed in a
step-wise fashion, where after each step new reagents
are added, or simultaneously, where all reagents are
added at the initial step, or partially step-wise and
10   partially simultaneous, where fresh reagent is added
after a given number of steps. If a method of strand
separation, such as heat, is employed which will
inactivate the agent for polymerization, as in the
case of a heat-labile enzyme, then it is necessary to
15   replenish the agent for polymerization after every
strand separation step. The simultaneous method may
be utilized when a number of purified components,
including an enzymatic means such as helicase, is
used for the strand separation step. In the
20   simultaneous procedure, the reaction mixture may
contain, in addition to the nucleic acid strand(s)
containing the desired sequence, the strand-
separating enzyme (e.g., helicase), an appropriate
energy source for the strand-separating enzyme, such
25   as ATP, the four nucleotides, the oligonucleotide
primers in molar excess, the PNA oligomers in molar
excess, and the inducing agent, e.g., Taq polymerase
or the Klenow fragment of E. coli DNA polymerase I.
If heat is used for denaturation in a simultaneous
30   process, a heat-stable inducing agent such as a
thermostable polymerase may be employed which will
operate at an elevated temperature, preferably 65° -
80° C, depending on the inducing agent, at which
temperature the nucleic acid will consist of single
35   and double strands in equilibrium. The upper

temperature will depend on the temperature at which the enzyme will degrade or the temperature above which an insufficient level of primer hybridization will occur. Each step of the process will occur

5 sequentially, notwithstanding the initial presence of all the reagents. Additional materials may be added as necessary. After the appropriate length of time has passed to produce the desired amount of the specific nucleic acid sequence, the reaction may be

10 halted by inactivating the enzymes in any known manner or by separating the components of the reaction.

The process of the present invention may be conducted continuously. In one embodiment of an

15 automated process, the reaction may be cycled through a denaturing region, a PNA oligomer reagent addition region, an oligonucleotide primer reagent addition region, and a reaction region. In another embodiment, the enzyme used for the synthesis of

20 primer extension products can be immobilized in a column. The other reaction components can be continuously circulated by a pump through the column and a heating coil in series; thus the nucleic acids produced can be repeatedly denatured without

25 inactivating the enzyme.

The steps of this process can be repeated indefinitely, being limited only by the amount of the oligonucleotide primers, the one or more PNA oligomers, the agent for polymerization and

30 nucleotides present. For detection, the number of cycles used is that required to produce a detectable amount, an amount which will depend, e.g., on the nature of the sample. For example, if the sample is pure, fewer cycles may be required than if it is a

complex mixture. If the sample is human genomic DNA, preferably the number of cycles is from about 10-30.

The amount of original nucleic acid remains constant in the entire process, because it is not

5 replicated. The amount of the long products increases linearly because they are produced only from the original nucleic acid. The amount of the specific sequence increases exponentially. Thus, the specific sequence will become the predominant

10 species.

When a single-stranded nucleic acid is utilized as the template, only one long product is formed per cycle.

The method herein may be utilized to clone a

15 particular nucleic acid sequence for insertion into a suitable expression vector. The vector may then be used to transform an appropriate host organism to produce the gene product of the sequence by standard method of recombinant DNA technology.

20 The amplification process herein may yield a mixture of nucleic acids, resulting from the original template nucleic acid, the expected target amplified products, and various background non-target products. The amplified product can also be a mixture, if the

25 original template DNA contains multiple target sequences, such as in a heterozygous diploid genome or when there is a family of related genes.

The primers herein may be modified to assist the rapid and specific cloning of the mixture of DNAs

30 produced by the amplification reaction. In such modification the same or different restriction sites are incorporated at the 5' ends of the primers to result in restriction sites at the two ends of the amplified product. When cut with the appropriate

35 enzymes, the amplified product can then be easily

- 30 -

inserted into plasmid or viral vectors and cloned.
This cloning allows the analysis or expression of
individual amplified products, not a mixture.

Although the same restriction site can be used
5   for both primers, the use of different sites allows
the insertion of the product into the vector with a
specific orientation and suppresses multiple
insertions as well as insertions arising from
amplifications based on only one of the two primers.
10   The specific orientation is useful when cloning into
single strand sequencing vectors, when single strand
hybridization probes are used, or when the cloned
product is being expressed.

One method to prepare the primers is to choose a
15   primer sequence which differs minimally from the
target sequence. Regions in which each of the
primers is to be located are screened for homology to
restriction sites appropriate to the desired vector.
For example, the target sequence "CAGTATCCGA . . ."
20   differs by only one base from one containing a BamHI
site. A primer sequence is chosen to match the
target exactly at its 3' end, and to contain the
altered sequence and restriction site near its 5' end
(for example, "CAGgATCCGA . . . ", where the lower
25   case letter symbolizes a mismatch with the target
sequence). This minimally altered sequence will not
interfere with the ability of the primer to hybridize
to the original target sequence and to initiate
polymerization. After the first amplification cycle
30   the primer is copied, becomes the target, and matches
exactly with new primers. After the amplification
process, the products are cleaved with the
appropriate restriction enzymes, optionally separated
from inhibitors of ligation, such as the nucleoside
35   triphosphates and salts, by passing over a desalting

- 31 -

column or molecular weight chromatography column, and
inserted by ligation into a cloning vector such as
bacteriophage M13. The gene may then be sequenced
and/or expressed using well known techniques.

5   The second method for preparing the primers
involves taking the 3' end of the primers from the
target sequence and adding the desired restriction
site(s) to the 5' end of the primer. For the above
example, a HindIII site could be added to make the

10   sequence "cgaagcttCAGTATCCGA . . .", where lower case
letters are as described above. The added bases
would not contribute to the hybridization in the
first cycle of amplification, but would match in
subsequent cycles. The final amplified products are

15   then cut with one or more restriction enzymes and
cloned and expressed as described above. The gene
being amplified may be, for example, human beta-
hemoglobin or the human HLA DQ, DR or DP-alpha and -
beta genes.

20   In addition, the process herein can be used for
in vitro mutagenesis. The oligodeoxyribonucleotide
primers need not be exactly complementary to the DNA
sequence which is being amplified. It is only
necessary that they be able to hybridize to the

25   sequence sufficiently well to be extended by the
polymerase enzyme or by whatever other inducing agent
is employed. The product of a polymerase chain
reaction wherein the primers employed are not exactly
complementary to the original template will contain

30   the sequence of the primer rather than the template,
thereby introducing an in vitro mutation. In further
cycles, this mutation will be amplified with
undiminished efficiency because no further mispaired
primings are required. The mutant thus produced may

35   be inserted into an appropriate v ctor by standard

- 32 -

molecular biological techniques and might confer
mutant properties on this vector such as the
potential for production of an altered protein.

   The process of making an altered DNA sequence as
5   described above could be repeated on the altered DNA
using different primers so as to induce further
sequence changes. In this way a series of mutated
sequences could gradually be produced wherein each
new addition to the series could differ from the last
10   in a minor way, but from the original DNA source
sequence in an increasingly major way. In this
manner changes could be made ultimately which were
not feasible in a single step due to the inability of
a very seriously mismatched primer to function.

15   In addition, the primer can contain as part of
its sequence a non-complementary sequence, provided
that a sufficient amount of the primer contains a
sequence which is complementary to the strand to be
amplified. For example, a nucleotide sequence which
20   is not complementary to the template sequence (such
as, e.g., a promoter, linker, coding sequence, etc.)
may be attached at the 5' end of one or both of the
primers, and thereby appended to the product of the
amplification process. After the extension primer is
25   added, sufficient cycles are run to achieve the
desired amount of new template containing the non-
complementary nucleotide insert. This allows
production of large quantities of the combined
fragments in a relatively short period of time (e.g.,
30   two hours or less) using a simple technique.

   If restriction site linkers are incorporated
into the primers, then the amplified double-stranded
products can be digested with the appropriate
restriction enzymes and ligated directly into a
35   vector for rapid cloning and sequencing. The plaques

- 33 -

containing the specific amplified target sequences can be identified by hybridizing plaque lift filters with a probe specific for the target sequence.

The method herein may also be used to enable
5   detection and/or characterization of specific nucleic acid sequences associated with infectious diseases, genetic disorders or cellular disorders such as cancer, e.g., oncogenes. Amplification is useful when the amount of nucleic acid available for
10 analysis is very small, as, for example, in the prenatal diagnosis of sickle cell anemia using DNA obtained from fetal cells or chorionic villi. Amplification is particularly useful if such an analysis is to be done on a small sample using non-
15 radioactive detection techniques which may be inherently insensitive, or where radioactive techniques are being employed but where rapid detection is desirable.

For purposes of this invention genetic diseases
20 may include specific deletions and/or mutations in genomic DNA from any organism, such as, e.g., sickle cell anemia, cystic fibrosis, alpha-thalassemia, beta-thalassemia, and the like. Sickle cell anemia can be readily detected via a RFLP-like analysis
25 following amplification of the appropriate DNA sequence by the present method. Alpha-thalassemia can be detected by the absence of a sequence, and beta-thalassemia can be detected by the presence of a polymorphic restriction site closely linked to a
30 mutation which causes the disease.

All of these genetic diseases may be detected by amplifying the appropriate sequence and analyzing it. In such a process, for example, a small sample of DNA from biological material, e.g., amniotic fluid or
35 chorionic villi containing a very low level of the

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- 34 -

desired sequence is amplified, cut with a restriction enzyme, and analyzed.

In another embodiment a small sample of DNA may be amplified to a convenient level and then a further
5   cycle of extension reactions performed wherein nucleotide derivatives which are readily detectable (such as ^{32}P-labeled or biotin-labeled nucleoside triphosphates) are incorporated directly into the final DNA product, which may be analyzed by
10   restriction and electrophoretic separation or any other appropriate method.

In a further embodiment, the nucleic acid may be exposed to a particular restriction endonuclease prior to amplification. Since a sequence which has
15   been cut cannot be amplified, the appearance of an amplified fragment, despite prior restriction of the DNA sample, implies the absence of a site for the endonuclease within the amplified sequence. The presence or absence of an amplified sequence can be
20   detected by an appropriate method.

In addition to detecting infectious diseases and pathological abnormalities in the genome of organisms, the process herein can also be used to detect DNA polymorphisms.
25   In a preferred embodiment of the present invention, the nucleic acid sequence which is to be amplified contains at least one sequence portion which is substantially repeated at least once within the nucleic acid sequence. This substantial
30   repetition encompasses both repetition of identical sequence portions and repetition of nearly-identical sequence portions. Nearly-identical portions are those portions which have 80% or greater sequence homology in a given region.

- 35 -

In another preferred embodiment of the present
invention, the PNA oligomer forms a duplex with a
portion of the nucleic acid sequence of interest,
which duplex has a melting temperature within the
5   range of 70° - 80° C, preferably 74° - 78° C. If the
nucleic acid sequence of interest contains a sequence
portion which is substantially repeated, then, in a
preferred embodiment, the PNA oligomer may be a
mixture of several PNA oligomers, each of which is
10   substantially complementary to one or more of the
identical or nearly-identical sequence portions.

In still another preferred embodiment of the
present invention, the PNA oligomer may be a pair of
PNA oligomers, at least a portion of the sequence of
15   each being complementary to the other member of the
pair.

Other features of the invention will become
apparent in the course of the following descriptions
of exemplary embodiments which are given for
20   illustration of the invention and are not intended to
be limiting thereof.

The PNA H-CCT(G/T)CCGGTGGTC(C/T)TC-NH2, a
composite of four PNA oligomers, was synthesized and
provided by Biosearch Division of PerSeptive
25   Biosytems. Bedford, MA. Oligonucleotide primers used
in the PCR reactions were synthesized by Research
Genetics (Huntsville, AL). The sequences of the
primers were 5'-GAAACTGGCCTCCAAACACTCCCCGCCG-3'
(forward primer), and 5'-
30   GTCTTGTTGGAGATGCACGTGCCCCTTGC-3'(reverse primer). The
forward primer was end-labeled with T4 polynucleotide
kinase and γ-^{32}P-ATP.

DNA was extracted from EDTA anticoagulated whole
blood using a PureGene DNA Isolation Kit (Gentra
35   Syst ms, Minneapolis, MN). Purified DNA from c ll

- 36 -

line K562 was purchased from Promega Corporation
(Madison, WI).

Amplification of the D1S80 locus was carried out
in a total volume of 12.5μl using 5ng of genomic DNA.
5   Each reaction contained 0.75 μM of each primer, 250
μM each of dCTP, dATP, dGTP, and TTP, and 1X
Strategene buffer (10mM Tris-HCl [pH 8.8], 50 mM KCl,
1.5 mM MgCl$_2$, and 0.01% (w/v) gelatin, and other
stabilizers not specified). The PCR reactions were
10   overlaid with 1 drop of mineral oil prior to addition
of 0.5 units of Stratagene Taq polymerase (La Jolla,
CA), the reaction mixtures were heated to 95° C for
4-10 minutes in a GTC-2 Genetic Thermal Cycler
(Precision Scientific, Chicago, IL). Subsequently, 5
15   cycles were carried out each consisting of 95° C for
1.25 minutes for denaturation, 67° C for 30 seconds
to allow primer annealing, and 4 minutes at 72° - 76°
C for primer extension, followed by 25 cycles each
consisting of 95° C for 1.25 minutes, 66° C for 30
20   seconds, and 72° - 76° C for 4 minutes. The final
extension was carried out for an additional 5
minutes.

Electrophoresis of the amplified DNA was carried
out on 5% Long Ranger denaturing gel (J.T. Baker) at
25   1000 V for 3.5 hr. Following electrophoresis, the
polyacrylamide gels were dried using a Savant Slab
Gel Dryer (Savant, Inc.). The amplification products
were visualized by autoradiography using Kodak XAR5
film and Biotech L-Plus intensifying screens (Fisher
30   Scientific).

Figure 5 is an autoradiograph illustrating the
results of the polymerase chain reaction obtained in
the absence of PNA oligomers and in the presence of
varying amounts of PNA oligomers. Lane 1 represents
35   the reaction detailed above run in the absence of the

- 37 -

four PNA oligomers. Lanes 2 to 5 represent the above reaction run in the presence of the four PNA oligomers at concentrations of 0.9 μM, 1.2 μM, 1.5 μM, and 1.8 μM, respectively. As is evidenced in the

5   autoradiograph, in the absence of PNA oligomers (Lane 1), differential amplification does occur. The smaller allele, located at the bottom half of Lane 1 of the autoradiograph, is much more strongly represented relative to the larger allele found in

10   the top half of Lane 1. This differential amplification can result in an erroneous analysis of the sample. In contrast, Lanes 2 to 5, which utilize different concentrations of the PNA oligomers, evidence a much reduced differential amplification.

15   To evaluate the general application of PNA for enhancing PCR at the D1S80 locus, 4 DNA specimens from routine paternity casework were randomly selected. Each was amplified with PNA (1.5 μM) and without PNA. All samples were prepared together from

20   the same master mix of reagents and cycled together in the same thermal cycler. Primer extension was carried out at 76°C. The results are shown in figure 6. Lanes 1, 3, 5, and 7 represent polymerase chain reaction run in the presence of PNA, while Lanes 2,

25   4, 6, and 8 represent experiments run in the absence of PNA. Each DNA specimen run with PNA demonstrated enhanced amplification, particularly of the larger allele.

         A PNA can be designed to be shorter than the

30   repeat so as to intentionally create gaps and allow read through by the polymerase. A shortened PNA might be useful for enhancing the amplification of loci consisting of conserved repeats.

         The foregoing description of preferred

35   embodiments of the invention has been presented for

purposes of illustration and description. It is not intended to be exhaustive or to limit the invention to the precise forms disclosed, and obviously many modifications and variations are possible in light of

5   the above teaching. The embodiment was chosen and described in order to best explain the principles of the invention and its practical application to thereby enable others skilled in the art to best utilize the invention in various embodiments and with

10 various modifications as are suited to the particular use contemplated. It is intended that the scope of the invention be defined by the claims appended hereto.

- 39 -

WHAT IS CLAIMED IS:

1.   A process for amplifying a nucleic acid
sequence of interest in a sample containing at
least one nucleic acid, comprising the steps of:
        treating the sample with at least one
PNA oligomer which binds within the nucleic acid
sequence to be amplified; and
        polymerizing a nucleic acid sequence
which is complementary to the nucleic acid
sequence of interest.

2.   The process according to claim 1,
further comprising the steps of:
        after said step of treating, annealing
the at least one PNA oligomer to said nucleic acid
sequence of interest;
        subsequently annealing the at least one
oligonucleotide primer to said nucleic acid
sequence of interest; and
        wherein said step of polymerizing
further comprises extending the oligonucleotide
primer so as to synthesize an extension product
employing said nucleic acid sequence of interest
as a template, such that the at least one PNA
oligomer is displaced from the nucleic acid
sequence of interest during synthesis of the
extension product.

3.   The process according to claim 2,
wherein said nucleic acid sequence of interest
includes a sequence portion which is substantially
repeated at least once within the nucleic acid
sequence of interest.

4.   The process according to claim 3,
wherein the at least one PNA oligomer comprises a
mixture of several PNA oligomers, each of which is
substantially complementary to said sequence portion.

1   5.   The process according to claim 2,
2   wherein said nucleic acid sequence of interest
3   includes a sequence portion which is repeated at
4   least once within the nucleic acid sequence of
5   interest.

1   6.   The process according to claim 2,
2   wherein during the step of annealing the at least
3   one PNA oligomer, said at least one PNA oligomer
4   forms a duplex with a portion of the nucleic acid
5   sequence of interest, said duplex having a melting
6   temperature within the range of 70° to 80° C.

1   7.   The process according to claim 6,
2   wherein said duplex has a melting temperature
3   within the range of 74° to 78° C.

1   8.   The process according to claim 2,
2   wherein the at least one PNA oligomer comprises a
3   pair of PNA oligomers, at least a portion of the
4   sequence of each of said pair being complementary
5   to the other.

1   9.   The process according to claim 2,
2   wherein said at least one PNA oligomer comprises a
3   PNA polymer containing 5 to 20 PNA monomers.

1   10. The process according to claim 9,
2   wherein said at least one PNA oligomer comprises a
3   PNA polymer containing 8 to 16 PNA monomers.

1   11. A method of amplifying a nucleic
2   acid sequence of interest in a sample containing
3   at least one nucleic acid, comprising:
4   (a)   treating the sample with
5   i)   an agent for polymerization,
6   ii) at least one oligonucleotide
7   primer which binds to a strand
8   of each different nucleic acid
9   sequence of interest,

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iii) at least one PNA oligomer
        which is substantially
        complementary to a portion of
        and which binds within the
        nucleic acid sequence of
        interest at a position
        different from the position at
        which the oligonucleotide
        primer binds, and
iv)   four different nucleoside
        triphosphates, forming a first
        mixture;

(b)   heating the first mixture to within
a first temperature range so as to denature the
nucleic acid and to separate any of the at least
one oligonucleotide primer and any of the at least
one PNA oligomer which is bound to the nucleic
acid, forming a second mixture;

(c)   cooling the second mixture to
within a second temperature range at which the at
least one PNA oligomer binds to the nucleic acid
sequence of interest, forming a third mixture;

(d)   bringing the third mixture to
within a third temperature range at which the at
least one oligonucleotide primer binds to the
nucleic acid sequence of interest and at which an
extension product is synthesized from the
oligonucleotide primer on each strand, provided
the nucleic acid sequence of interest is present,
said synthesis employing the nucleic acid as a
template, and whereby the at least one PNA
oligomer is displaced from the nucleic acid
sequence of interest during said synthesis.

12. The method according to claim 11, comprising repeating steps (b), (c), and (d) at least one time.

13. The method according to claim 11, further comprising the step of

(e) treating the product of step (d) under denaturing conditions to separate the extension product from said template.

14. The method according to claim 13, further comprising the step of

(f) detecting the presence of any of said nucleic acid sequence of interest.

15. The method according to claim 11, wherein the nucleic acid sequence of interest includes a sequence portion which is substantially repeated at least once within the nucleic acid sequence of interest.

16. The method according to claim 15, wherein the at least one PNA oligomer comprises a mixture of several PNA oligomers, each of which is substantially complementary to said sequence portion.

17. The method according to claim 11, wherein said nucleic acid sequence of interest includes a sequence portion which is repeated at least once within the nucleic acid sequence of interest.

18. The method according to claim 11, wherein during step (c) the at least one PNA oligomer forms a duplex with a portion of the nucleic acid sequence of interest, said duplex having a melting temperature within the range of 70° to 80° C.

1   19. The method according to claim 18,
2   wherein said duplex has a melting temperature
3   within the range of 74° to 78° C.

1   20. The method according to claim 11,
2   wherein the at least one PNA oligomer comprises a
3   pair of PNA oligomers, at least a portion of the
4   sequence of each of said pair being complementary
5   to the other.

          21. The method according to claim 11,
wherein the first temperature range is a range of
80° to 100° C.

1   22. The method according to claim 11,
2   wherein the second temperature range is a range of
3   70° to 80° C.

1   23. The method according to claim 22,
2   wherein the second temperature range is a range of
3   72° to 78° C.

1   24. The method according to claim 11,
2   wherein the third temperature range is a range of
3   40° to 80° C.

1   25. The method according to claim 11,
2   wherein said at least one PNA oligomer comprises a
3   PNA polymer containing 5 to 20 PNA monomers.

          26. The method according to claim 25,
wherein said at least one PNA oligomer comprises a
PNA polymer containing 8 to 16 PNA monomers.

1   27. The method according to claim 11,
2   wherein, while performing steps (c) and (d), the
3   temperature is continuously varied.

1   28. The method according to claim 11,
2   wherein, during each of steps (c) and (d), the
3   temperature is maintained at a substantially
4   constant value for an interval of time.

1   29. The method according to claim 11,
2   wherein step (d) comprises the steps of:

3   (d1) bringing the third mixture to
4 within a fourth temperature range which is within
5 said third temperature range and at which the at
6 least one oligonucleotide primer binds to the
7 nucleic acid sequence of interest, forming a
8 fourth mixture;
9   (d2) heating the fourth mixture to
10 within a fifth temperature range which is within
11 said third temperature range and at which said
12 extension product is synthesized from the
13 oligonucleotide primer on each strand, whereby the
14 at least one PNA oligomer is displaced from the
15 nucleic acid sequence of interest, forming a fifth
16 mixture.

1   30. The method according to claim 29,
2 comprising repeating steps (b), (c), and (d) at
3 least one time.

1   31. The method according to claim 29,
2 further comprising the step of
3   (e) treating the product of step (d)
4 under denaturing conditions to separate the
5 extension product from said template.

1   32. The method according to claim 31,
2 further comprising the step of
3   (f) detecting the presence of any of
4 said nucleic acid sequence of interest.

1   33. The method according to claim 29,
2 wherein the first temperature range is a range of
3 80° to 100° C.

1   34. The method according to claim 29,
2 wherein the second temperature range is a range of
3 70° to 80° C.

1   35. The method according to claim 34,
2 wherein the second temperature range is a range of
3 72° to 78° C.

- 45 -

1   36. The method according to claim 29,
2   wherein the fourth temperature range is a range of
3   40° to 72° C.

1   37. The method according to claim 29,
2   wherein the fifth temperature range is a range of
3   60° to 80° C.

1   38. A kit for detecting at least one nucleic
2   acid sequence of interest in a sample, comprising:
3   (a) a container containing at least one
4   oligonucleotide primer for each strand of each
5   nucleic acid sequence of interest, each of said at
6   least one oligonucleotide primer being
7   substantially complementary to a strand of each
8   nucleic acid sequence of interest;
9   (b) a container containing at least one
10   PNA oligomer which is substantially complementary
11   to a portion of and which binds within each
12   nucleic acid sequence of interest at a position
13   different from the position at which the at least
14   one oligonucleotide primer binds; and
15   (c) a container containing each of four
16   different nucleoside triphosphates.

1   39. The kit according to claim 38, further
2   comprising:
3   (d) a container containing an agent for
4   polymerization.

1   40. The kit according to claim 38 wherein
2   said nucleic acid of interest includes a sequence
3   portion which is substantially repeated at least
4   once within the nucleic acid sequence of interest.

1   41. The kit according to claim 40, wherein
2   the at least one PNA oligomer comprises a mixture
3   of several PNA oligomers, each of which is
4   substantially complementary to said sequence
5   portion.

- 46 -

1   42.   The kit according to claim 38 wherein
2   said nucleic acid sequence of interest includes a
3   sequence portion which is repeated at least once
4   within the nucleic acid sequence of interest.

1   43.   The kit according to claim 38, wherein
2   the at least one PNA oligomer forms a duplex with
3   a portion of the nucleic acid sequence of
4   interest, said duplex having a melting temperature
5   within the range of 70° to 80° C.

1   44.   The kit according to claim 43, wherein
2   said duplex has a melting temperature within the
3   range of 74° to 78° C.

1   45.   The kit according to claim 38, wherein
2   the at least one PNA oligomer comprises a pair of
3   PNA oligomers, at least a portion of the sequence
4   of each of said pair being complementary to the
5   other.

1   46.   The kit according to claim 38, wherein
2   said at least one PNA oligomer comprises a PNA
3   polymer containing 5 to 20 PNA monomers.

1   47.   The kit according to claim 46, wherein
2   said at least one PNA oligomer comprises a PNA
3   polymer containing 8 to 16 PNA monomers.

1   48.   A process for cloning into a vector a
2   nucleic acid sequence of interest in a sample
3   containing at least one nucleic acid, comprising:
4   (a)   treating the sample with
5   i)   an agent for polymerization,
6   ii)   at least one oligonucleotide
7   primer containing a
8   restriction site at its 5' end
9   and which is substantially
10   complementary to and binds to
11   a strand of each different

12   nucleic acid sequence of

13   interest,

14   iii)   at least one PNA oligomer

15   which is substantially

16   complementary to a portion of

17   and which binds within the

18   nucleic acid sequence of

19   interest at a position

20   different from the position at

21   which the oligonucleotide

22   primer binds, and

23   iv)   four different nucleoside

24   triphosphates, forming a first

25   mixture;

26   (b)   heating the first mixture to within

27   a first temperature range so as to denature the

28   nucleic acid and to separate any of the at least

29   one oligonucleotide primer and any of the at least

30   one PNA oligomer which is bound to the nucleic

31   acid, forming a second mixture;

32   (c)   cooling the second mixture to

33   within a second temperature range at which the at

34   least one PNA oligomer binds to the nucleic acid,

35   forming a third mixture;

36   (d)   bringing the third mixture to

37   within a third temperature range at which the at

38   least one oligonucleotide primer binds to the

39   nucleic acid sequence of interest and at which an

40   extension product is synthesized from the

41   oligonucleotide primer on each strand, provided

42   the nucleic acid sequence of interest is present,

43   said synthesis employing the nucleic acid as a

44   template, and whereby the at least one PNA

45   oligomer is displaced from the nucleic acid

46   sequence of interest during said synthesis

47   (e)   adding to the product of step (d) a
48   restriction enzyme which cleaves at said
49   restriction site to thereby obtain cleaved
50   products; and
51   (f)   ligating the cleaved products into
52   at least one cloning vector.

1   49.   The process according to claim 48,
2   comprising repeating steps (b), (c), and (d) at
3   least one time.

1   50.   The process according to claim 48,
2   wherein said nucleic acid sequence of interest
3   includes a sequence portion which is substantially
4   repeated at least once within the nucleic acid
5   sequence of interest.

1   51.   The process according to claim 50,
2   wherein the at least one PNA oligomer comprises a
3   mixture of several PNA oligomers, each of which is
4   substantially complementary to said sequence
5   portion.

1   52.   The process according to claim 48,
2   wherein said nucleic acid sequence of interest
3   includes a sequence portion which is repeated at
4   least once within the nucleic acid sequence of
5   interest.

1   53.   The process according to claim 48,
2   wherein during step (c) the at least one PNA
3   oligomer forms a duplex with a portion of the
4   nucleic acid sequence of interest, said duplex
5   having a melting temperature within the range of
6   70° to 80° C.

1   54.   The process according to claim 53,
2   wherein said duplex has a melting temperature
3   within the range of 74° to 78° C.

1   55.   The process according to claim 48,
2   wherein the at least one PNA oligomer comprises a

3 pair of PNA oligomers, at least a portion of the
4 sequence of each of said pair being complementary
5 to the other.

1   56.   The process according to claim 48,
2 wherein the first temperature range is a range of
3 80° to 100° C.

1   57.   The process according to claim 48,
2 wherein the second temperature range is a range of
3 70° to 80° C.

1   58.   The process according to claim 57,
2 wherein the second temperature range is a range of
3 72° to 78° C.

1   59.   The process according to claim 48,
2 wherein the third temperature range is a range of
3 40° to 80° C.

1   60.   The process according to claim 48,
2 wherein said at least one PNA oligomer comprises a
3 PNA polymer containing 5 to 20 PNA monomers.

1   61.   The process according to claim 60,
2 wherein said at least one PNA oligomer comprises a
3 PNA polymer containing 8 to 16 PNA monomers.

1   62.   The process according to claim 48,
2 wherein, while performing steps (c) and (d), the
3 temperature is continuously varied.

1   63.   The process according to claim 48,
2 wherein, during each of steps (c) and (d), the
3 temperature is maintained at a substantially
4 constant value for an interval of time.

1   64.   The process according to claim 48,
2 wherein step (d) comprises the steps of:
3   (d1) bringing the third mixture to
4 within a fourth temperature range which is within
5 said third temperature range and at which the at
6 least one oligonucleotide primer binds to the

7   nucleic acid sequence of interest, forming a

8   fourth mixture;

9   (d2)   heating the fourth mixture to

10   within a fifth temperature range which is within

11   said third temperature range and at which said

12   extension product is synthesized from the

13   oligonucleotide primer on each strand, whereby at

14   least one PNA oligomer is displaced from the

15   nucleic acid sequence of interest, forming a fifth

16   mixture.

1   65.   The process according to claim 64,

2   wherein the first temperature range is a range of

3   80° to 100° C.

1   66.   The process according to claim 64,

2   wherein the second temperature range is a range of

3   70° to 80° C.

1   67.   The process according to claim 66,

2   wherein the second temperature range is a range of

3   72° to 78° C.

1   68.   The process according to claim 64,

2   wherein the fourth temperature range is a range of

3   40° to 72° C.

1   69.   The process according to claim 64,

2   wherein the fifth temperature range is a range of

3   60° to 80° C.

1   70.   An amplified nucleic acid sequence of

2   interest produced by a process comprising the

3   steps of:

4   (a)   treating the sample with

5   i)   an agent for polymerization,

6   ii)   at least one oligonucleotide

7   primer which binds to a strand

8   of each nucleic acid sequence

9   of interest,

10   iii)   at least one PNA oligomer
11   which is substantially
12   complementary to a portion of
13   and which binds within the
14   nucleic acid sequence of
15   interest at a position
16   different from the position at
17   which the at least one
18   oligonucleotide primer binds,
19   and
20   iv)   four different nucleoside
21   triphosphates, forming a first
22   mixture;
23   (b) heating the first mixture to within
24   a first temperature range so as to denature the
25   nucleic acid and to separate any of the at least
26   one oligonucleotide primer and any of the at least
27   one PNA oligomer which is bound to the nucleic
28   acid, forming a second mixture;
29   (c) cooling the second mixture to within
30   a second temperature range at which the at least
31   one PNA oligomer binds to the nucleic acid
32   sequence of interest, forming a third mixture;
33   (d) bringing the third mixture to
34   within a third temperature range at which the at
35   least one oligonucleotide primer binds to the
36   nucleic acid sequence of interest and at which an
37   extension product is synthesized from the
38   oligonucleotide primer on each strand, provided
39   the nucleic acid sequence of interest is present,
40   said synthesis employing the nucleic acid as a
41   template, and whereby the at least one PNA
42   oligomer is displaced from the nucleic acid
43   sequence of interest during said synthesis.

- 52 -

1   71. The amplified nucleic acid sequence
2   according to claim 70, comprising repeating steps
3   (b), (c), and (d) at least one time.
1   72. The amplified nucleic acid sequence
2   according to claim 70, further comprising the step
3   of   (e) treating the product of step (d)
4   under denaturing conditions to separate the
5   extension product from said template.
1   73. The amplified nucleic acid sequence
2   according to claim 72, further comprising the step
3   of   (f) detecting the nucleic acid sequence
4   of interest.
1   74. The amplified nucleic acid sequence
2   according to claim 70, wherein said nucleic acid
3   sequence of interest includes a sequence portion
4   which is substantially repeated at least once
5   within the nucleic acid sequence of interest.
1   75. The amplified nucleic acid sequence
2   according to claim 74, wherein the at least one
3   PNA oligomer comprises a mixture of several PNA
4   oligomers, each of which is substantially
5   complementary to said sequence portion.
1   76. The amplified nucleic acid sequence
2   according to claim 70, wherein said nucleic acid
3   sequence of interest includes a sequence portion
4   which is repeated at least once within the nucleic
5   acid sequence of interest.
1   77. The amplified nucleic acid sequence
2   according to claim 70, wherein during step (c) the
3   at least one PNA oligomer forms a duplex with a
4   portion of the nucleic acid sequence of interest,
5   said duplex having a melting temperature within
6   the range of 70° to 80° C.
1   78. The amplified nucleic acid sequence
2   according to claim 77, wherein said duplex has a

3   melting temperature within the range of 74° to 78°
4   C.

1   79.   The amplified nucleic acid sequence
2   according to claim 70, wherein the at least one
3   PNA oligomer comprises a pair of PNA oligomers, at
4   least a portion of the sequence of each of said
5   pair being complementary to the other.

1   80.   The amplified nucleic acid sequence
2   according to claim 70, wherein the first
3   temperature range is a range of 80° to 100° C.

1   81.   The amplified nucleic acid sequence
2   according to claim 70, wherein the second
3   temperature range is a range of 70° to 80° C.

1   82.   The amplified nucleic acid sequence
2   according to claim 81, wherein the second
3   temperature range is a range of 72° to 78° C.

1   83.   The amplified nucleic acid sequence
2   according to claim 70, wherein the third
3   temperature range is a range of 40° to 80° C.

1   84.   The amplified nucleic acid sequence
2   according to claim 70, wherein said at least one
3   PNA oligomer comprises a PNA polymer containing 5
4   to 20 PNA monomers.

1   85.   The amplified nucleic acid sequence
2   according to claim 84, wherein said at least one
3   PNA oligomer comprises a PNA polymer containing 8
4   to 16 PNA monomers.

1   86.   The amplified nucleic acid sequence
2   according to claim 70, wherein, while performing
3   steps (c) and (d), the temperature is continuously
4   varied.

1   87.   The amplified nucleic acid sequence
2   according to claim 70, wherein, wherein, during
3   each of steps (c) and (d), the temperature is

- 54 -

4   maintained at a substantially constant value for

5   an interval of time.

1   88. The amplified nucleic acid sequence

2   according to claim 70, wherein step (d) comprises

3   the steps of:

4   (d1) bringing the third mixture to

5   within a fourth temperature range which is within

6   said third temperature range and at which the at

7   least one oligonucleotide primer binds to the

8   nucleic acid sequence of interest, forming a

9   fourth mixture;

10   (d2) heating the fourth mixture to

11   within a fifth temperature range which is within

12   said third temperature range and at which said

13   extension product is synthesized from the

14   oligonucleotide primer on each strand, whereby the

15   at least one PNA oligomer is displaced from the

16   nucleic acid sequence of interest, forming a fifth

17   mixture.

1   89. The method according to claim 88,

2   comprising repeating steps (b), (c), and (d) at

3   least one time.

1   90. The method according to claim 88,

2   further comprising the step of

3   (e) treating the product of step (d)

4   under denaturing conditions to separate the

5   extension product from said template.

1   91. The method according to claim 89,

2   further comprising the step of

3   (f) detecting the presence of any of

4   said nucleic acid sequence of interest.

1   92. The amplified nucleic acid sequence

2   according to claim 88, wherein the first

3   temperature range is a range of 80° to 100° C.

1   93.   The amplified nucleic acid sequence
2   according to claim 88, wherein the second
3   temperature range is a range of 70° to 80° C.
1   94.   The amplified nucleic acid sequence
2   according to claim 93, wherein the second
3   temperature range is a range of 72° to 78° C.
1   95.   The amplified nucleic acid sequence
2   according to claim 88, wherein the fourth
3   temperature range is a range of 40° to 72° C.
1   96.   The amplified nucleic acid sequence
2   according to claim 88, wherein the fifth
3   temperature range is a range of 60° to 80° C.
1   97.   A method for replicating a target
2   nucleic acid strand with a polymerase to produce a
3   complementary nucleic acid strand, comprising:
4   (A)   providing a peptide nucleic acid
5   (PNA) which,
6   (1)   is substantially complementary
7   in sequence to nucleobases in
8   at least a portion of the
9   target strand,
10   (2)   anneals with said portion of
11   the target strand, and
12   (3)   when annealed with said
13   portion of the target strand,
14   does not serve as a
15   replication initiation site
16   for the polymerase; and
17   (B)   annealing the PNA to the target
18   strand prior to initiating
19   replication thereby to enhance
20   replication by the polymerase
21   during production of the
22   complementary nucleic acid strand.

- 56 -

1   98.   The method according to claim 97,
2   wherein step (A) comprises providing a mixture of
3   PNAs, wherein the nucleobase sequence of at least
4   one of the PNAs in the mixture is complementary in
5   sequence to said portion of the target strand.
1   99.   The method according to claim 97,
2   wherein the PNA provided in step (A) comprises 5
3   to 20 nucleobases complementary in sequence to
4   said portion of the target strand.
1   100. The method according to claim 99,
2   wherein the PNA provided in step (A) comprises 8
3   to 16 nucleobases complementary in sequence to
4   said portion of the target strand.
1   101. The method according to claim 97,
2   wherein the target strand comprises a multiplicity
3   of nucleobase sequence repeats.
1   102. The method according to claim 97,
2   wherein the polymerase is a DNA polymerase.
1   103. The method according to claim 102,
2   wherein the DNA polymerase is a thermostable DNA
3   polymerase.
1   104. The method according to claim 102,
2   further comprising the step of annealing an
3   oligonucleotide primer to a second nucleobase
4   sequence of the target strand thereby to produce a
5   replication initiation site for the polymerase.
1   105. The method according to claim 103,
2   further comprising the step of annealing an
3   oligonucleotide primer to a second nucleobase
4   sequence of the target strand thereby to produce a
5   replication initiation site for the polymerase.
1   106. A method for replicating a target
2   nucleic acid strand with a thermostable DNA
3   polymerase to produce a complementary nucleic acid
4   strand, comprising:

- 57 -

       5   (A)   providing a peptide nucleic acid

       6   (PNA) which,

       7   (1)   is substantially complementary

       8   in sequence to nucleobases in

       9   at least a portion of the

      10   target strand,

      11   (2)   anneals with said portion of

      12   the target strand, and

      13   (3)   when annealed with said

      14   portion of the target strand,

      15   does not serve as a

      16   replication initiation site

      17   for the polymerase; and

      18   (B)   annealing the PNA to the target

      19   strand prior to initiating

      20   replication by the polymerase

      21   thereby to enhance replication by

      22   the polymerase during production of

      23   the complementary nucleic acid

      24   strand.

       1   107. The method according to claim 106,

       2   wherein step (A) comprises providing a mixture of

       3   PNAs, wherein the nucleobase sequence of at least

       4   one of the PNAs in the mixture is complementary in

       5   sequence to said portion of the target strand.

       1   108. The method according to claim 106,

       2   wherein the PNA provided in step (A) comprises 5

       3   to 20 nucleobases complementary in sequence to

       4   said portion of the target strand.

       1   109. The method according to claim 108,

       2   wherein the PNA provided in step (A) comprises 8

       3   to 16 nucleobases complementary in sequence to

       4   said portion of the target strand.

- 58 -

1   110. The method according to claim 106,
2   wherein the target strand comprises a multiplicity
3   of nucleobase sequence repeats.
1   111. The method according to claim 106,
2   further comprising the step of:
3   (C) annealing an oligonucleotide primer
4   to a second nucleobase sequence of the target
5   strand thereby to produce a replication initiation
6   site for the polymerase.
1   112. A method for replicating a target
2   nucleic acid strand with a polymerase comprising:
3   (A) providing a target nucleic acid
4   strand having a multiplicity of
5   nucleobase sequence repeats;
6   (B) annealing a PNA to the target
7   strand which:
8   (1) is substantially complementary
9   in sequence to at least a
10   portion of one of the repeats;
11   (2) anneals with the at least a
12   portion of one of the repeats
13   thereby to form a target
14   strand-PNA complex; and
15   (3) when annealed with the at
16   least a portion of one of the
17   repeats, does not serve as a
18   replication initiation site
19   for the polymerase; and
20   (C) incubating the complex in the
21   presence of a polymerase, such that
22   the polymerase enhances replication
23   of the target strand as compared
24   with when the target strand is not
25   complexed with the PNA.

- 59 -

1   113. The method according to claim 112,
2   wherein step (B) comprises providing a mixture of
3   PNAs, wherein the nucleobase sequence of at least
4   one of the PNAs in the mixture is complementary in
5   sequence to said at least a portion of one of the
6   repeats.

1   114. The method according to claim 112,
2   wherein the PNA provided in step (B) comprises 5
3   to 20 nucleobases complementary in sequence to
4   said at least a portion of one of the repeats.
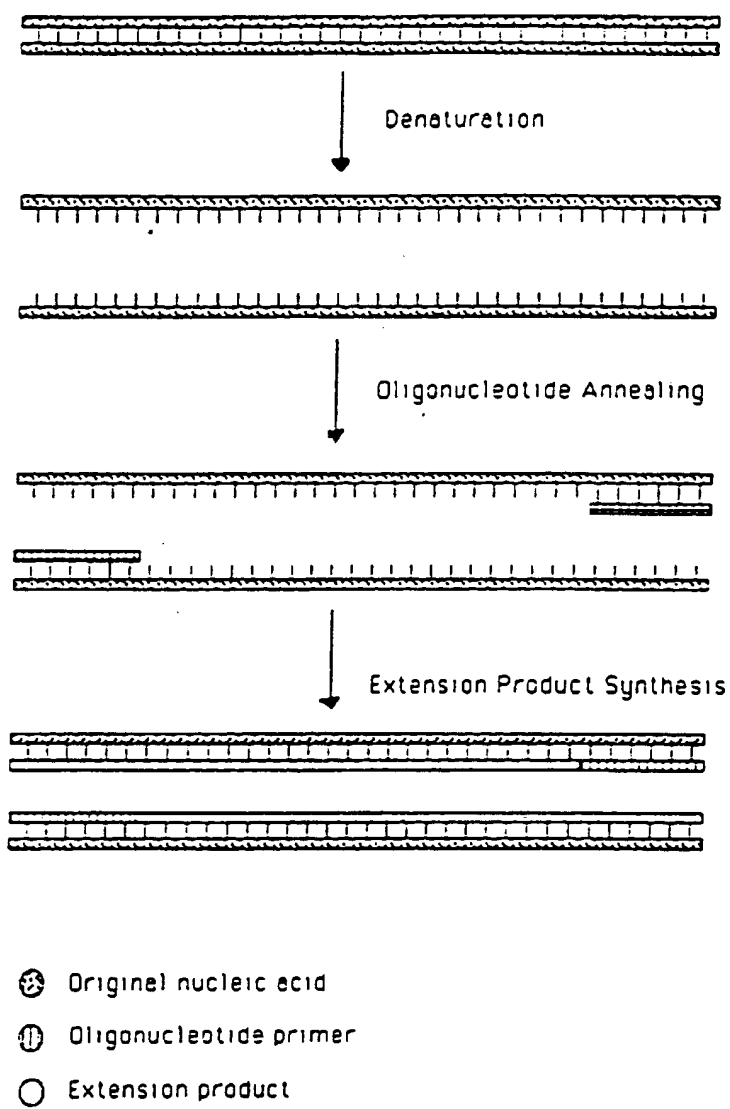
1   115. The method according to claim 114,
2   wherein the PNA provided in step (B) comprises 8
3   to 16 nucleobases complementary in sequence to
4   said at least a portion of one of the repeats.

1   116. The method according to claim 112,
2   wherein the polymerase is a DNA polymerase.

1   117. The method according to claim 116,
2   wherein the DNA polymerase is a thermostable DNA
3   polymerase.

1   118. The method according to claim 112
2   further comprising the step of:
3   (D)   annealing a primer to a second
4   sequence of the target strand
5   thereby to produce a replication
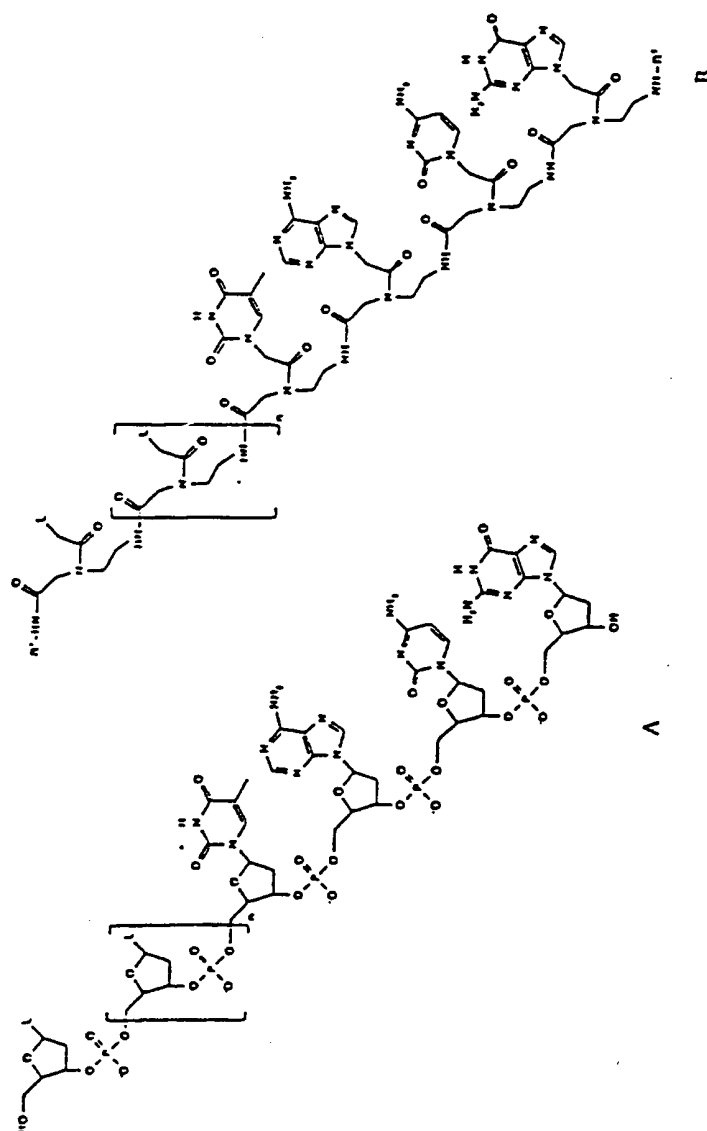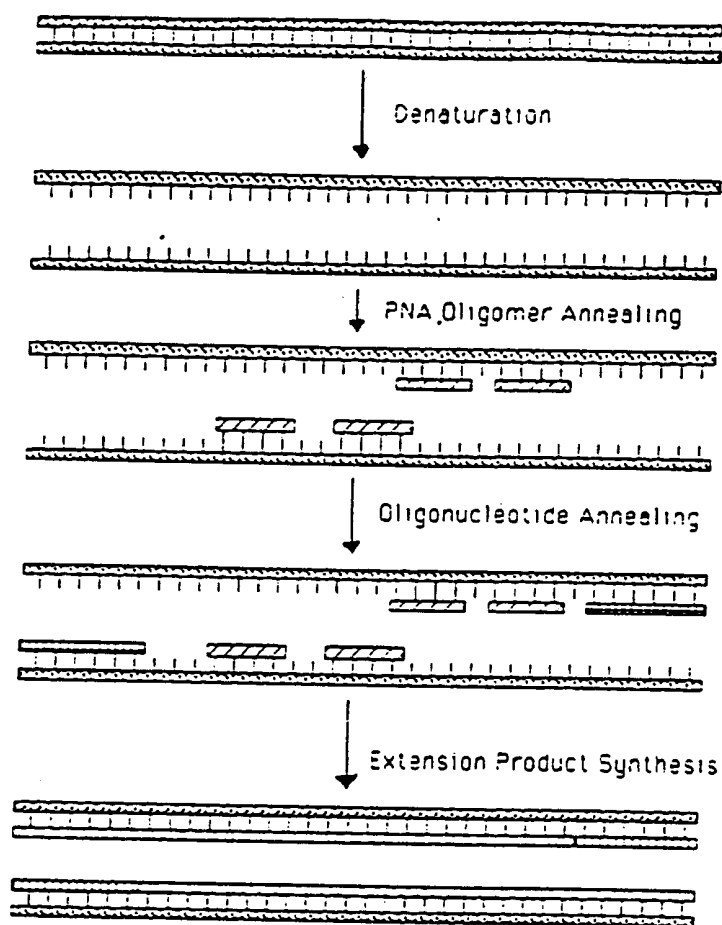6   initiation site for the polymerase.

Denaturation

Oligonucleotide Annealing

Extension Product Synthesis
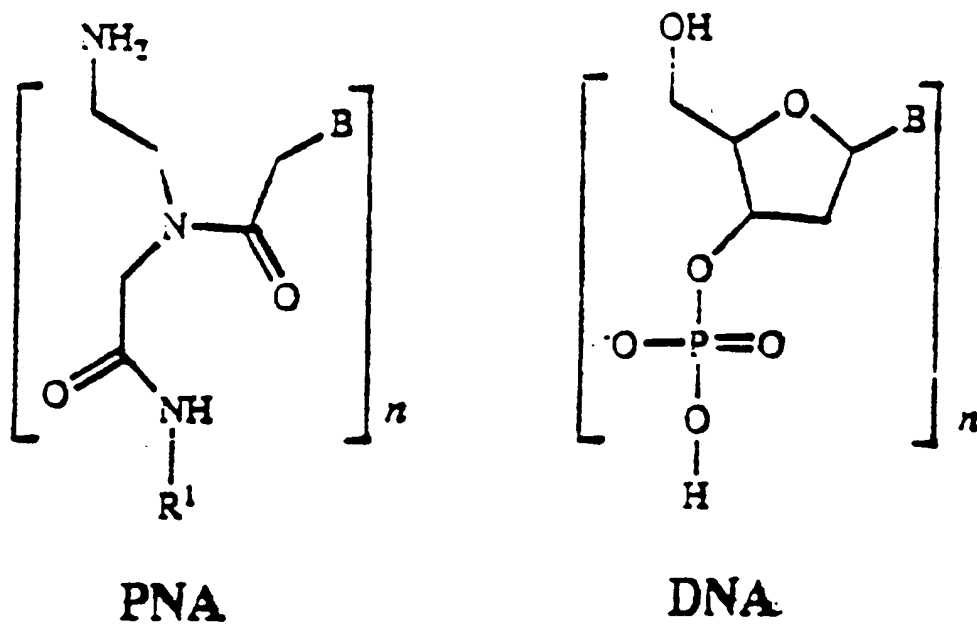
⊛ Original nucleic acid

⊕ Oligonucleotide primer

○ Extension product

Figure 1.

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

6 of 6

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/13345

A.	CLASSIFICATION OF SUBJECT MATTER

IPC(5)   :C12Q 1/68, 1/70; C12P 19/34; C07H 21/04; A61K 48/00
US CL   :435/6, 5, 91.2; 536/24.5, 44
According to International Patent Classification (IPC) or to both national classification and IPC

B.	FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :   435/6, 5, 91.2; 536/24.5, 44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C.	DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Nucleic Acids Research, Volume 21, Number 23, issued 02 June 1993, H. Orum et al, "Single base pair mutation analysis by PNA directed PCR clamping", pages 5332-5336, especially pages 5332-5332.	38-47
X	Innis et al, "PCR Protocols, A Guide to Methods and Applications" published 1990 by Academic Press (N.Y.), pages 13-20, see Figure 1.	70-88, 92-96
X, P	WO, A, 94/28171 (PNA DIAGNOSTICS) 08 December 1994, especially page 27.	38-47
P, A	WO, A, 95/08556 (AMERSHAM INTERNATIONAL PLC) 30 March 1995, especially pages 3-7.	1-118

[X] Further documents are listed in the continuation of Box C.   [ ]   See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
04 JANUARY 1996	0 1 FEB 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer DIANNE REES
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Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Proceedings of the National Academy of Science, Volume 90, issued March 1993, D.Y. Cherny et al, "DNA unwinding upon strand-displacement binding of a thymine-substituted polyamide to double-stranded DNA", pages 1667-1670, especially page 1670.	1-118

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, PROMT, JAPIO, BIOBUSINESS, CABA, WPIDS, BIOTECHDS, BIOSIS, EMBASE, MEDLINE, DISSABS, SCISEARCH, AIDSLINE, CANCERLIT, TOXLIT, TOXLINE, USPATFULL

search terms: PNA, peptide nucleic acids, PCR, amplification, helix unwinding, helix destabilization, DNA melting, repeats, hairpins, or palindromes, loops, cloning using PNAs.